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Alzheimer Precursor Protein Interaction with the Nogo-66 Receptor Reduces Amyloid-β Plaque Deposition

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Pathophysiologic hypotheses for Alzheimer's disease (AD) are centered on the role of the amyloid plaque Aβ peptide and the mechanism of its derivation from the amyloid precursor protein (APP). As part of the disease process, an aberrant axonal sprouting response is known to occur near Aβ deposits. A Nogo to Nogo-66 receptor (NgR) pathway contributes to determining the ability of adult CNS axons to extend after traumatic injuries. Here, we consider the potential role of NgR mechanisms in AD. Both Nogo and NgR are mislocalized in AD brain samples. APP physically associates with the NgR. Overexpression of NgR decreases Aβ production in neuroblastoma culture, and targeted disruption of NgR expression increases transgenic mouse brain Aβ levels, Aβ plaque deposition, and dystrophic neurites. Infusion of a soluble NgR fragment reduces Aβ levels, amyloid plaque deposits, and dystrophic neurites in a mouse transgenic AD model. Changes in NgR level produce parallel changes in secreted APPα and Aβ, implicating NgR as a blocker of secretase processing of APP. The NgR provides a novel site for modifying the course of AD and highlights the role of axonal dysfunction in the disease.

Key words: **Alzheimer's disease; β-amyloid plaque; Nogo; transgenic mice; Nogo-66 receptor; gene targeting; amyloid precursor protein; APP**

Introduction

Amyloid plaques and neurofibrillary tangles are the principal pathologic hallmarks that accompany neuronal loss in the dementia of Alzheimer's disease (AD) (Hardy and Selkoe, 2002; Selkoe and Schenk, 2003). The amyloid Aβ peptide is derived proteolytically from amyloid precursor protein (APP) and is the major constituent of the amyloid plaque in AD. Although the rate of Aβ peptide release from APP is implicated in AD pathophysiology, there is less certainty regarding which forms of Aβ result in neuronal dysfunction and by what mechanism this occurs. The transformation of monomeric Aβ to large amyloid plaque deposits proceeds through several intermediate steps, and intermediate forms may be causative in the neuronal dysfunction of AD (Klein, 2002; Walsh et al., 2002b). Aβ peptides have been shown to interact with several macromolecules that might potentially contribute to pathology. Such Aβ-binding proteins include, but are not limited to, the receptor for advanced glycation end products (Yang et al., 1996), the low-affinity NGF receptor p75-NTR (Kuner et al., 1998) and nicotinic acetylcholine receptors (Wang et al., 2000; Dineley et al., 2001; Nagele et al., 2002). The cellular effects of Aβ application have included cell death, altered synaptic transmission, stimulation of neurite outgrowth, and inhibition of neurite outgrowth (Walsh et al., 2002a; Kamenetz et al., 2003). It remains unclear which cellular effects depend on specific molecular interactions and are most relevant to the ability of Aβ to cause dementia.

There are several observations that connect APP and Aβ to axonal mechanisms. Many amyloid plaques, the so-called "neuritic plaques," exhibit a cluster of dystrophic neurites surrounding their edge (Lombardo et al., 2003). This suggests that aberrant, ineffective sprouting mechanisms are activated in the vicinity of Aβ deposits. Recent data have demonstrated that local Aβ deposits are dependent on axonal input, implying that the principal source of Aβ is peptide released from the presynaptic ending of the axon (Lazarov et al., 2002; Sheng et al., 2002). In brain trauma, APP accumulation in transected axons is one of the better markers of injured axons (Otsuka et al., 1991; Scott et al., 1991; Gentleman et al., 1993). Furthermore, APP has been implicated as an adaptor protein for kinesin I-based axonal transport (Kamal et al., 2001). Most recently, intriguing studies have demonstrated physical interactions between Reticulon family proteins (including Nogo) and BACE1 (β-secretase activity of the β-site APP-cleaving enzyme), one of the proteases responsible for Aβ production from APP (He et al., 2004). For these reasons, we considered the potential connection between those molecular processes that regulate axonal sprouting in the adult CNS and the APP/Aβ pathologic process. One pathway implicated in adult axonal CNS sprouting is the Nogo/Nogo receptor (NgR) pathway (Chen et al., 2000; GrandPre et al., 2000, 2002; Prinjha et al., 2000; Fournier et al., 2001; Kim et al., 2003, 2004; Li and Strittmatter, 2003; McGee and Strittmatter, 2003; Lee et al., 2004; Li et al.,

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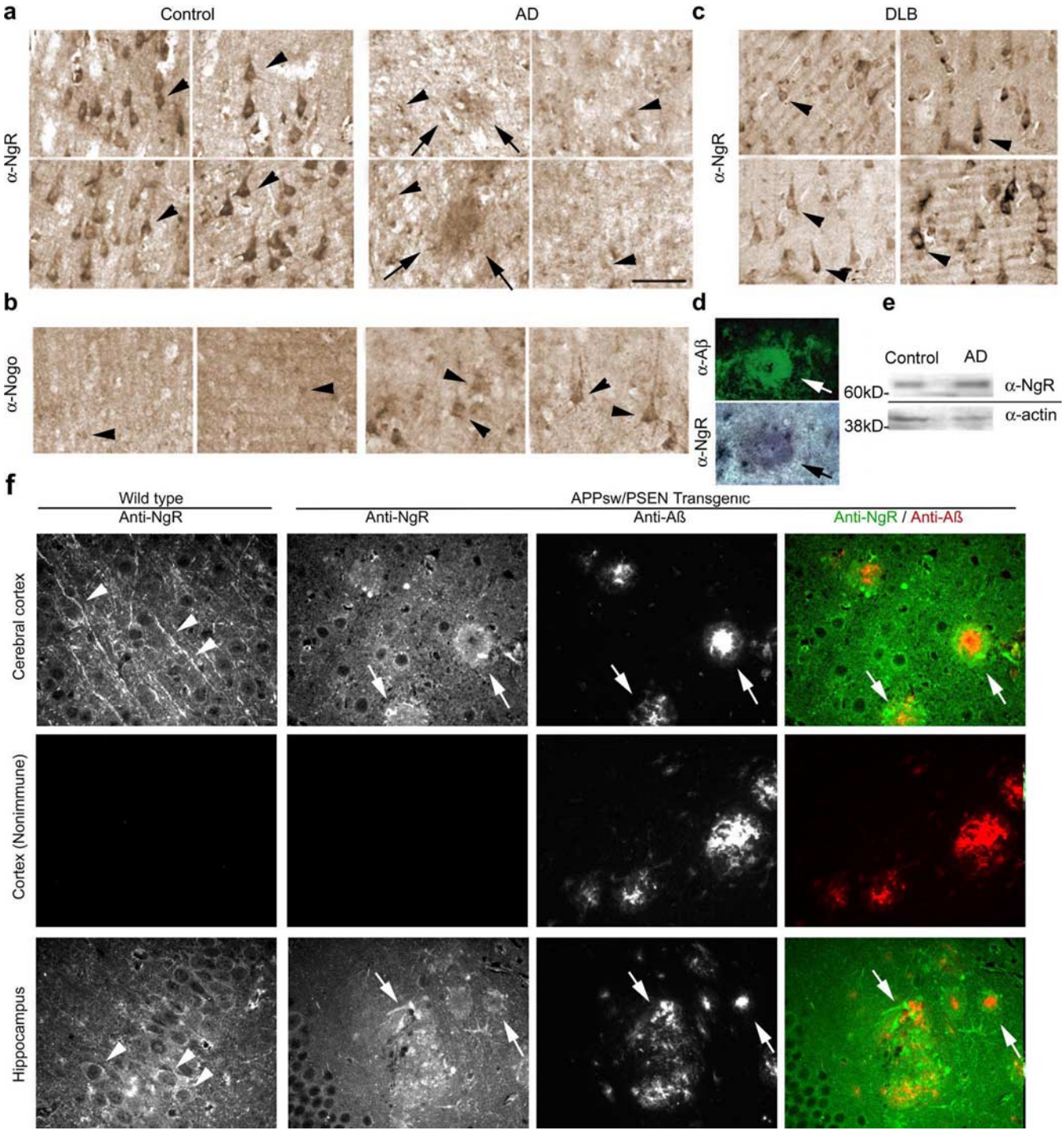

Figure 1. Altered Nogo and NgR localization in human AD brain. ***a–c***, Human brain samples from age-matched control samples, AD, or diffuse Lewy body dementia (DLB) were processed for anti-NgR or anti-Nogo-A immunohistology as indicated. All samples are from the hippocampus. Note the reduced cellular NgR localization in AD and the enhanced cellular Nogo localization in the AD samples (arrowheads, cellular staining). Some amyloid plaques are NgR immunoreactive (arrows). ***d***, The colocalization of NgR and Aβ in plaques is shown in higher magnification at the bottom right (arrows). ***e***, Anti-NgR immunoblot demonstrates the specificity of the anti-NgR antibody and the unchanged NgR level in the AD brain compared with controls. Two additional control and two additional AD cases had staining patterns similar to that shown here. Anti-actin immunoblot demonstrates similar protein loading. ***f***, Sections of cerebral cortex or hippocampus from wild-type or APPswe/PSEN transgenic mice of 9 months age were stained with anti-NgR or nonimmune IgG (green in merged image) and anti-Aβ antibody (6E10, red in merged image). Brains were fixed in paraformaldehyde, sectioned in paraffin, and treated with formic acid before staining. In wild-type brain, axonal profiles are detected in the cerebral cortex, and cell soma are stained in the hippocampus (arrowheads). In transgenic brain, cellular and axonal staining is reduced, but periplaque NgR immunoreactivity (arrows) is detected.

2004). We find that NgR interacts with APP and Aβ to limit Aβ accumulation *in vivo*.

Materials and Methods

Human brain tissue samples were obtained from the National Institutes of Health-supported Harvard Brain Tissue Resource Center with no personal identifying information. Anti-Nogo-A and anti-NgR rabbit antibodies and immunohistological methods have been described previously (Wang et al., 2002), as have NgR expression vectors, alkaline phosphatase (AP)-Nogo-66 protein, and the purified soluble function-blocking NgR ectodomain [NgR(310)ecto-Fc] (GrandPre et al., 2000; Fournier et al., 2001, 2002; Lee et al., 2004; Li et al., 2004). The anti-NgR antibody raised

in goat was from R & D Systems (Minneapolis, MN) (AF1440). The AP-Aβ and AP-APP proteins were produced by the same method as AP-Nogo-66. The binding of AP fusion proteins to transfected COS-7 cells has been described previously (GrandPre et al., 2000; Fournier et al., 2001, 2002; Li et al., 2004). N2A neuroblastoma cells stably expressing the Swedish form of APP (APPswe) and the 369 anti-C-terminal-APP antibody were generous gifts from S. S. Sisodia (University of Chicago, Chicago, IL) (Thinakaran et al., 1996). The anti-N-terminal-APP 5228 antibody, the anti-$A\beta_{1-17}$ 6E10 antibody, and the anti-C-terminal-APP 5352 antibody were obtained from Chemicon (Temecula, CA). The anti-Aβ 4G8 antibody and the anti-C-terminal-APP 51–2700 antibodies were from Signet Laboratories (Dedham, MA) and Zymed Laboratories (South San Francisco, CA), respectively. The anti-secretet APP (SAPP)βswe-specific 6A1 antibody was from Immunobiological Laboratories (Gunma, Japan). $A\beta_{1-40}$ and $A\beta_{40-1}$ with an N-terminal biotin tag were synthesized at the W. M. Keck Biotechnology facility at Yale University.

For covalent cross-linking studies, adult rat brain was homogenized in PBS plus protease inhibitor mixture (Roche Products, Welwyn Garden City, UK). The particulate fraction was collected by centrifugation at 100,000 × *g* for 20 min. Membranes were resuspended in PBS (1 ml/gm brain wet weight) and incubated with 3 mM *bis*(sulfosuccinimidyl) suberate (BS^3) for 1 h at 4°C. Unreacted cross-linker was quenched by addition of Tris-HCl, pH 7.6, to 0.1 M. Particulate material was again collected by centrifugation, and then membrane protein was solubilized with 1% Triton X-100. The detergent extract was subjected to nonimmune IgG or anti-NgR (1D9) immunoprecipitation using protein A/G Sepharose (Pierce, Rockford, IL) and analyzed by anti-NgR plus anti-APP immunoblot.

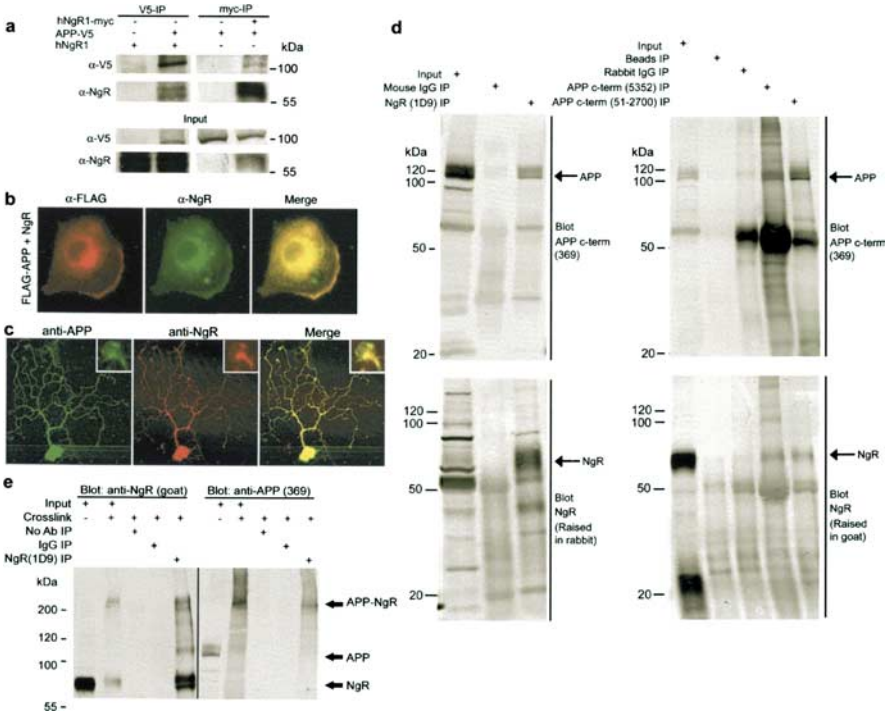

Figure 2. Physical interaction of NgR and APP. ***a***, Epitope-tagged version of human APP and NgR were expressed in HEK-293T cells alone or in combination. Immunoprecipitates of APP show detectable NgR, and immunoprecipitates of NgR exhibit APP immunoreactivity. Twelve percent of the input protein is loaded in the bottom panels. ***b***, Transfected COS-7 cells expressing Flag-APP (red) and NgR (green) were examined by double-labeled immunohistochemistry. ***c***, APP and NgR localization in neurons. Mouse postnatal day 5 DRG neurons were stained with anti-APP (green) and anti-NgR antibodies (red). The insets show higher magnification of one growth cone. ***d***, Rat brain homogenates were immunoprecipitated with 1D9 (monoclonal anti-NgR), control IgG, 5352 (anti-C-terminal-APP), and 51–2700 (anti-C-terminal-APP). Coimmunoprecipitation of APP and NgR was detected by immunoblotting with the indicated antibodies. Seven percent of the input protein is loaded in the indicated lanes. ***e***, Particulate fractions of adult rat brain were treated for 1 h at 4°C with 3 mM BS^3. Detergent solubilized protein was immunoprecipitated with anti-NgR antibody as in ***d***. The immunoprecipitates were examined for NgR and APP immunoreactivity. Note the formation of a high-molecular-weight complex that is immunoreactive for both NgR and APP.

The generation of $NgR^{-/-}$ mice has been described previously (Kim et al., 2004). In this line, exon II containing the entire mature coding region is deleted and no NgR protein is produced. In the studies here, the NgR mutant allele from a 129/sv embryonic stem cell was backcrossed to C57BL/6J for four to six generations before breeding with APPswe/presenilin-1 (PSEN-1)(ΔE9) transgenic mice. In all experiments, littermate mice carrying one APPswe/PSEN-1(ΔE9) transgene and either heterozygous or homozygous for the NgR null mutation were compared with one another. Transgenic APPswe/PSEN-1(ΔE9) (Borchelt et al., 1997; Jankowsky et al., 2003) mice were from The Jackson Laboratory (Bar Harbor, ME) (stock #04462) and were obtained on a mixed strain background as described by the provider.

Aβ ELISA assays were performed according to the instructions of the manufacturer (Biosource, Camarillo, CA). Aβ plaques in parasagittal sections of 4% paraformaldehyde-fixed brain were detected immunohistologically with anti-$A\beta_{1-17}$ 6E10 antibody after 0.1 M formic acid treatment for antigen recovery. Plaque area was quantitated using NIH Image as a percentage of total cerebral cortical area for three sections from each animal. Neuritic dystrophy was visualized by staining with monoclonal anti-synaptophysin antibodies (Sigma, St. Louis, MO) in parasagittal paraffin-embedded sections. The area of cerebral cortex and hippocampus occupied by clusters of dystrophic neurites was measured as a percentage of total area, using the outer border of increased staining by the same method as for Aβ plaque load. For analysis of sAPPα and Aβ levels from brain extracts, forebrain was extracted with 0.1 M formic acid, neutralized with Tris, and clarified by centrifugation at 10,000 × *g*.

To administer NgR(310)ecto-Fc protein to mice, animals were anesthetized with isoflurane and oxygen, and a burr hole was drilled in the skull. A cannula (Alzet brain infusion kit II; Alza, Palo Alto, CA) was introduced into the right lateral ventricle at stereotaxic coordinates 0.6 mm posterior and 1.2 mm lateral to bregma and 2.0 mm deep to the pial surface. The cannula was held in place with cyanoacrylate, and the catheter was attached to a subcutaneous osmotic minipump (Alzet 2004; Alza). The pump delivered 0.25 μl/h for 28 d of a 1.2 mg/ml solution of NgR(310)ecto-Fc or rat IgG in PBS. Pumps were replaced after 28 d and connected to the same cannula.

Results

Altered NgR and Nogo subcellular localization in AD

As a first direct step toward considering a role for the Nogo-NgR system in AD, human brain sections from AD cases and controls were examined histologically for Nogo-A and NgR localization (Fig. 1*a*,*b*,*d*). Tissue from the hippocampus and Brodmann's area 20/36 were examined in six control and six AD cases. In the control adult human brain, Nogo-A immunoreactivity is detectable in a diffuse granular pattern in the neuropil of these brain regions with little cellular staining. Here, we have focused on gray matter in which a majority of Nogo is neuronal, not oligodendrocytic in origin, as in white matter (Wang et al., 2002). In all of the AD cases, there is a dramatic shift of Nogo-A immunoreactivity to cell bodies (Fig. 1*b*, arrowheads). NgR localization in the AD brain is shifted in the opposite manner (Fig. 1*a*). In control cases, the highest concentration of the NgR protein is found in cell soma (arrowheads). Both the pattern of staining in human sam-

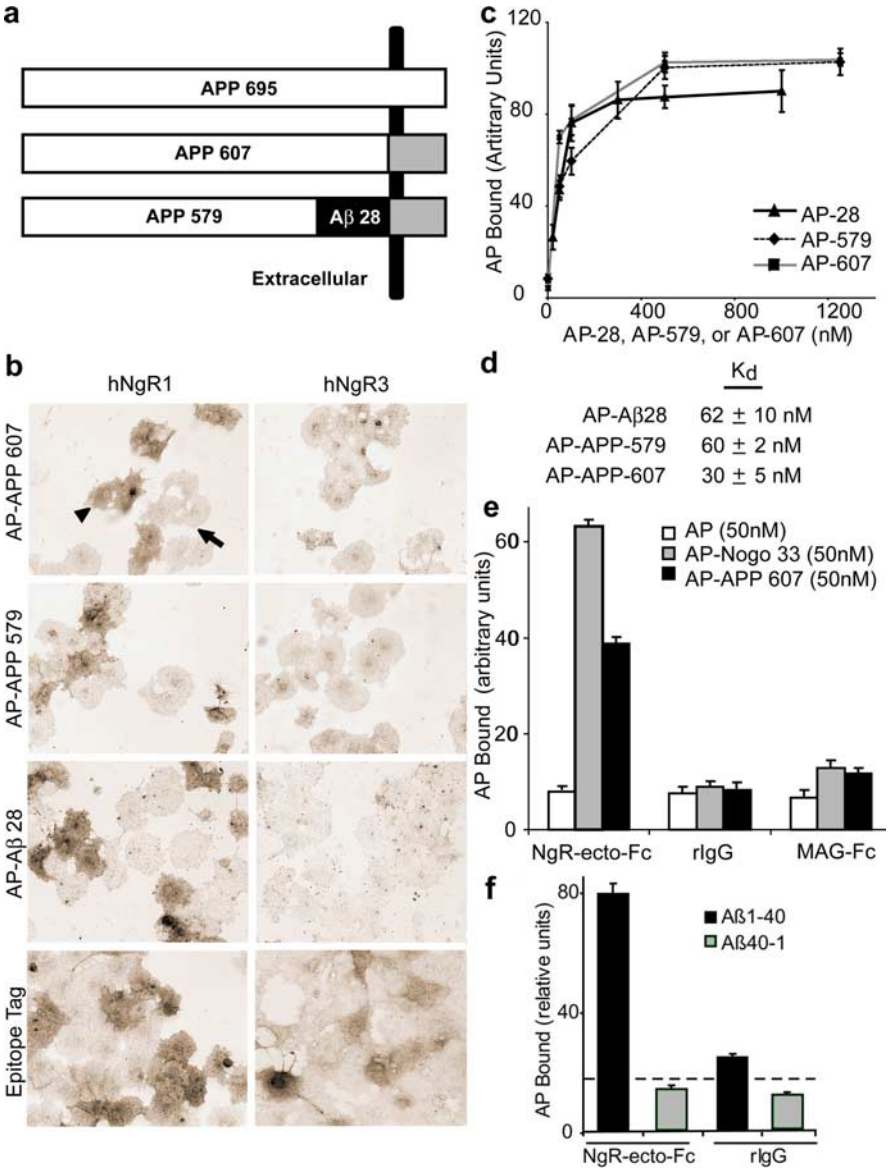

Figure 3. APP ectodomain binds to NgR1-expressing cells. ***a***, A schematic illustrates the cellular orientation of APP and the fragments that were used to express AP fusion proteins. ***b***, COS-7 cells were cultured with transfection of human NgR1 or human NgR3 expression plasmid, and their respective expressions were verified by epitope-tag immunohistology. Fifty nanomolar solutions of AP-APP607, AP-APP579, and Aβ_{28}-AP were allowed to bind to transfected cells for 2 h before washing, fixation, heat inactivation of endogenous AP, and enzymatic detection of bound fusion protein. Note the dark reaction product by arrowhead derived in cultures expressing NgR1 but not NgR3. An untransfected cell is labeled by the arrow. This is one of five experiments with similar results. ***c***, ***d***, Binding of AP-Aβ_{28}, APP579, and APP607 to NgR1-expressing COS-7 cells from an experiment as in ***a*** was measured as a function of ligand concentration from 5 to 1250 nM. Data are means ± SEM from one of three experiments with similar results. The calculated K_d is noted in ***d***. ***e***, Purified NgR-ecto(310)-Fc, MAG-Fc, or BSA (100 ng of each protein) was coated onto microtiter wells. After blockade of nonspecific sites with excess BSA, the wells were exposed to 50 nM AP, AP-Nogo 33, or AP-APP607. Data are the means ± SEM from three similar experiments. Note the selective binding of either Nogo(1–33) or APP607 to NgR-coated wells. ***f***, The binding of 100 nM biotin-Aβ_{1-40} or biotin-Aβ_{40-1} to immobilized NgR(344)ecto-Fc or IgG (100 ng of each protein) was detected by retention of streptavidin-AP.

ples and the previous murine studies suggest that the cellular NgR immunoreactivity is neuronal (Wang et al., 2002). Four AD brain samples exhibit little cellular NgR staining but diffuse immunoreactivity in the neuropil and in plaque-like deposits. Neither NgR nor Nogo-A colocalizes with neurofibrillary tangles or dystrophic neurites recognized by an antibody directed against hyperphosphorylated Tau protein (data not shown). Specificity of staining was demonstrated by antigen blockade and by the recognition of a single immunoreactive protein on immunoblots of human samples (Fig. 1*e* and data not shown). By immunoblot analysis, the total level of NgR is not altered in AD samples (Fig. 1*e*). The absence of NgR staining in cell bodies is not attributable to a complete absence of neurons, as can be appreciated clearly in the adjacent sections stained with Nogo-A antibodies (Fig. 1, compare *a*, *b*). Altered NgR localization is not observed in a different dementing illness, diffuse Lewy body dementia (Fig. 1*c*). In addition to the shift of NgR out of the cell soma, the protein is concentrated in amyloid plaques (Fig. 1*a*,*d*, arrows). Double immunohistochemistry for Aβ and NgR demonstrates their colocalization in these deposits (Fig. 1*d*). These findings suggest that the Nogo/NgR pathway might have either a primary or secondary role in AD pathology.

We also examined NgR localization in a transgenic mouse model of AD, the APPswe/PSEN-1(ΔE9) double-transgenic mice (Borchelt et al., 1997; Jankowsky et al., 2003). NgR-immunoreactive processes are prominent in the cerebral cortex (Fig. 1*f*, arrowheads, top left), whereas neuronal cell bodies are stained in the CA3 region of the hippocampus of wild-type mice (Fig. 1*f*, arrowheads, bottom left). NgR staining of cell bodies is less prominent in the transgenic mice (arrowheads), and axons are not readily apparent in the APPswe/PSEN-1(ΔE9) cerebral cortex. A fraction of the protein is detected at the border of amyloid plaques (Fig. 1*f*, arrows). NgR staining is most prominent at the circumference of intense Aβ deposits (Fig. 1*f*, merged image). The transgenic mouse model confirms the existence of altered NgR localization in AD.

APP interacts with NgR in transfected cells and brain

Based on these observations of Nogo and NgR localization, we considered whether there might be direct interactions between these proteins and APP. Epitope-tagged versions of the proteins were expressed in human embryonic kidney HEK-293T cells and immunoprecipitation studies were performed. APP specifically associates with NgR (Fig. 2*a*) but not with Nogo-A in these studies (data not shown). If APP and NgR are to associate in living cells, then at least a portion of the proteins must colocalize at the subcellular level. Both proteins are known to be associated with lipid rafts (Fournier et al., 2002; Ehehalt et al., 2003), and double-immunohistochemical studies reveal a nearly complete overlap of the distribution for two proteins in transfected cells and primary neurons (Fig. 2*b*,*c*). The physical association of endogenous NgR and APP was also examined in rat brain homogenates. Immunoprecipitates of APP contain NgR immunoreactivity, and anti-NgR isolates possess APP

protein detected with different APP antibodies (Fig. 2*d*). To verify that the physical association of NgR with APP occurs within intact tissue and is not dependent on detergent solubilization, covalent cross-linking studies of rat brain membranes were performed (Fig. 2*e*). Approximately half of NgR is covalently coupled by BS^3 treatment into a high-molecular-weight complex with an apparent molecular weight (M_r) of 200–250 kDa. The high M_r NgR-immunoprecipitable complex is also strongly immunoreactive for APP. The apparent molecular mass of this complex is greater than would be predicted for a 1:1 NgR/APP complex and may reflect aberrantly slow migration of a cross-linked complex or the presence of a more complicated structure including two copies of NgR or additional protein species. We conclude that a fraction of APP and NgR are physically associated within neurons.

We sought to determine which region of APP might interact with NgR. Because NgR is a glycophosphatidylinositol (GPI)-anchored protein, any direct interaction must be mediated by the APP extracellular (ecto) domain. We created fusion proteins containing various portions of the APP ectodomain fused to AP. At nanomolar concentrations, the ecto-APP-AP protein binds to NgR-expressing COS cells but not to vector-transfected COS cells or to COS cells expressing the related protein NgR3 (Fig. 3). The APP extracellular domain is cleaved by β-secretase enzyme into two fragments. As AP fusion proteins, the sAPPβ fragment (APP597) and $Aβ_{1–28}$ ectodomain fragment both exhibit affinity for NgR (Fig. 3*b–d*). Thus, the physical interaction of APP with NgR occurs through both amino and carboxyl segments of the ectodomain of APP. Furthermore, both β-secretase substrates (APP607) and β-secretase products (APP597 and $Aβ_{1–28}$) can interact with NgR.

The association of APP and NgR might be direct or indirect. To distinguish these possibilities, we immobilized purified NgR protein and examined the binding of APP-AP ligand in the absence of other cellular constituents (Fig. 3*e*). The APP ligand clearly binds to NgR under these minimal conditions, demonstrating a direct association of the two proteins.

The AP fusion method required a truncated $Aβ_{1–28}$ fragment to maintain protein ligand solubility. We used synthetic forms of $Aβ_{1–40}$ to verify that the endogenous peptide also interacts with NgR. In the ELISA format, the interaction of biotin-$Aβ_{1–40}$ with NgR is easily measured above background (Fig. 3*f*). In contrast, the reverse $Aβ_{40–1}$ peptide does not interact with immobilized NgR. $Aβ_{1–40}$ binding to cell surface NgR was examined using biotinylated peptide, fluorescent avidin, and cell sorting (Fig. 4). The binding of $Aβ_{1–40}$ to COS-7 cells is dramatically enhanced by NgR expression, and this binding is suppressed by anti-NgR an-

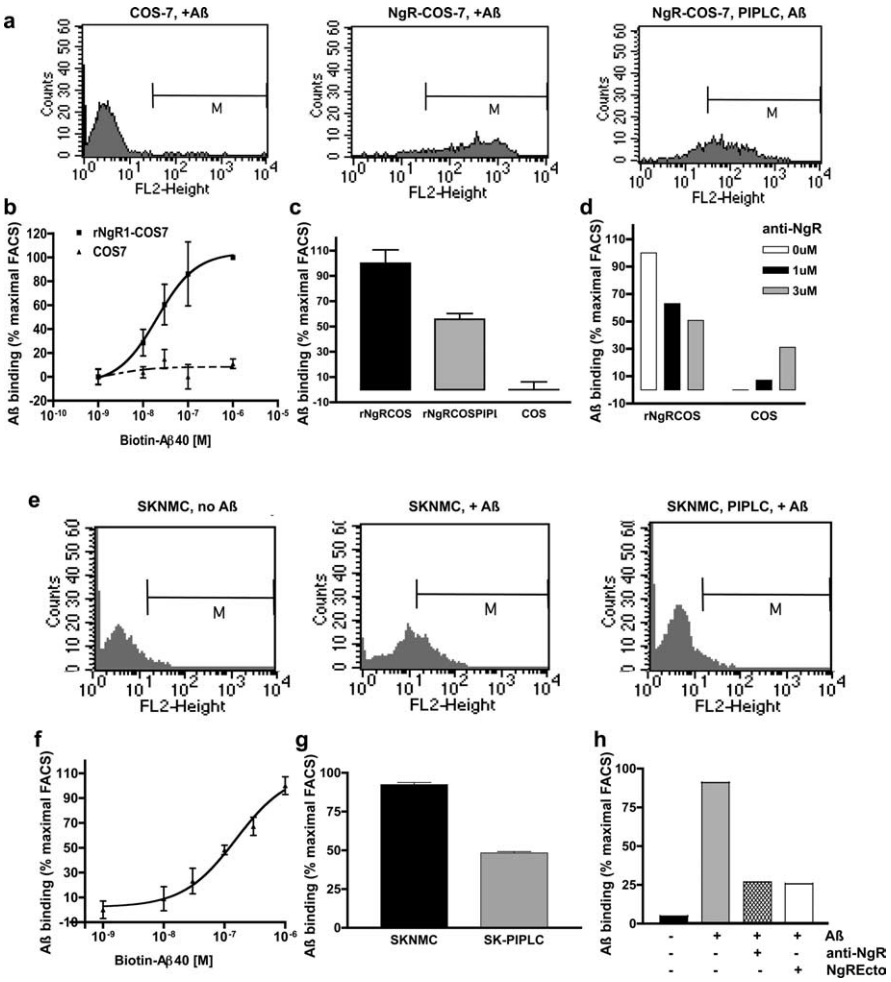

Figure 4. $Aβ_{1–40}$ binds to NgR-expressing cells. ***a–d***, Binding of 100 nM biotinylated $Aβ_{1–40}$ to COS-7 cells transfected with vector or rat NgR expression plasmid. Bound Aβ was detected by fluorescence-activated cell sorting (FACS) after incubation with Alexa488-conjugated streptavidin (***a***). Some cells were treated with 1 U of PI-PLC for 1 h at 37°C to release GPI-anchored proteins from the cell surface immediately before binding (***a***, ***c***). The gating criterion for the quantitation of binding is illustrated by a bar, M. The dose dependence for binding was assessed with the indicated concentrations of Aβ (***b***). The NgR1 antibody 7E11 was added before biotinylated 100 nM Aβ to displace binding from NgR-expressing COS-7 cells (***d***). ***e–h***, The fluorescence-activated cell sorting method was used to detect biotin-$Aβ_{1–40}$ binding to human neuroblastoma SKNMC cells (***e***). $Aβ_{1–40}$ bound dose dependently (***f***) and the binding was diminished after PI-PLC treatment (***e***, ***g***). ***h***, Biotinylated $Aβ_{1–40}$ was displaced from SKNMC by anti-NgR antibodies (2E10, 1 μM) and by soluble NgR(344)ecto-Fc (1 μM). Data are means ± SEM from three to six experiments with similar results.

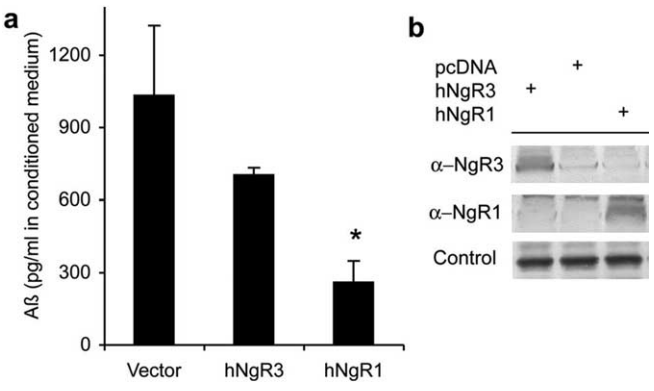

Figure 5. NgR expression decreases Aβ formation by neuroblastoma cells. Neuroblastoma N2A cells stably expressing APPswe (Thinakaran et al., 1996) were transiently transfected with control plasmid or vectors directing the expression of hNgR3 or hNgR1. ***a***, $Aβ_{1–40}$ ELISA was performed on conditioned medium. *$p \leq 0.025$; the decrease in Aβ is significant and represents data from four independent transfections. ***b***, N2A cell lysate was examined by immunoblot with the indicated antibodies to verify expression.

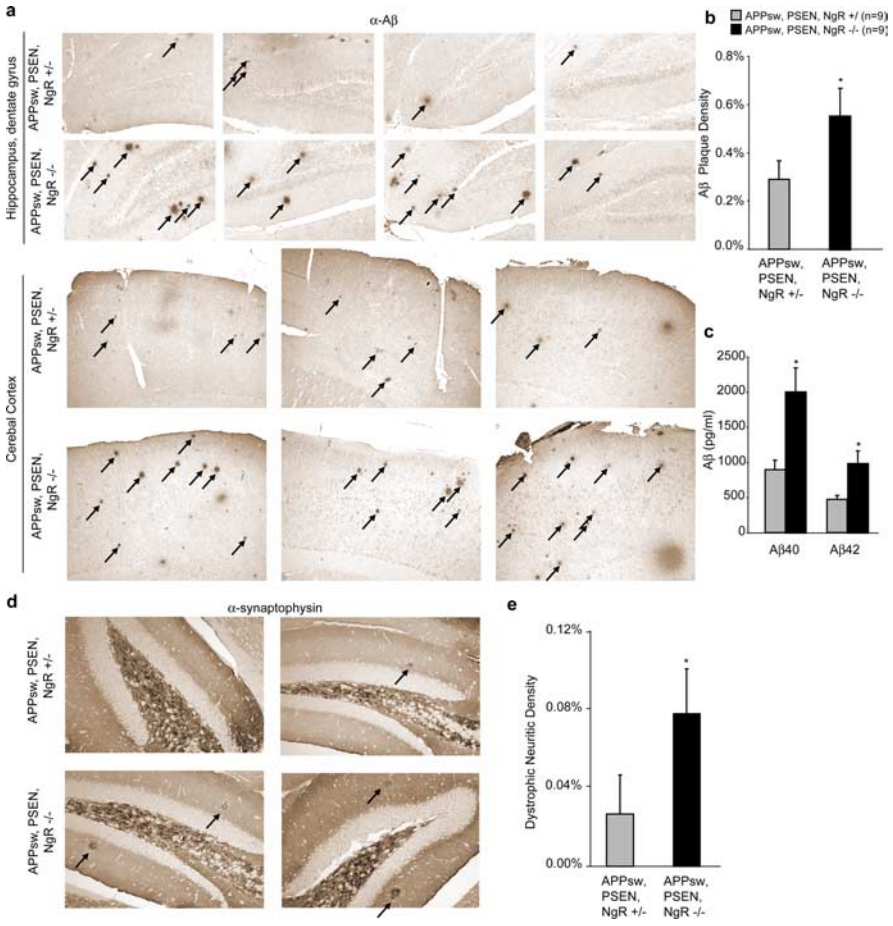

Figure 6. Enhanced Aβ and neuritic dystrophy in FAD transgenic mice lacking NgR. Mice coexpressing human APPswe/PSEN-1(ΔE9) and heterozygous for a null allele of NgR were intercrossed with $NgR^{-/-}$ mice. At 6 months of age, littermate-matched mice expressing APPswe/PSEN-1(ΔE9) and either $NgR^{+/-}$ or $NgR^{-/-}$ were killed. Brain tissue was analyzed by immunohistochemistry and ELISA. ***a***, ***b***, Arrows indicate examples of plaque deposits in hippocampal dentate gyrus and cerebral cortex. Plaque densities were quantified from nine mice in each group and are significantly different (*$p \leq 0.05$, Student's *t* test). ***c***, $A\beta_{1-40}$ and $A\beta_{1-42}$ levels were assessed by ELISA and are significantly different (*$p \leq 0.04$ level, Student's *t* test) for nine mice of each genotype. ***d***, ***e***, Anti-synaptophysin-immunoreactive dystrophic neurites are illustrated (arrows, ***d***). The percentage of area occupied by synaptophysin-positive dystrophic neurites is reported in ***e***. Data are means ± SEM from nine mice per group. *$p < 0.05$, Student's *t* test; the decrease in dystrophic neurites is significant.

tibody or by phosphatidylinositol-specific phospholipase C (PI-PLC) treatment to cleave the GPI-anchored NgR protein from the cells. The binding of Aβ to SKNMC neuroblastoma cells containing endogenous NgR is also suppressed by anti-NgR antibodies, PI-PLC treatment, and soluble NgR-ectodomain decoy receptor. Thus, NgR provides a high-affinity binding site for APP and Aβ.

NgR overexpression reduces Aβ production in neuroblastoma cells

One of the critical steps in AD is the proteolytic production of Aβ from APP. The effect of NgR on this processing was assessed in N2A neuroblastoma cells stably expressing APPswe (Fig. 5*a*,*b*). Conditioned medium from these cells contains a significant level of Aβ (Thinakaran et al., 1996). Expression of endogenous NgR in the N2A cell line is low and undetectable by immunoblots. Overexpression of NgR, but not NgR3, in the APPswe-N2A cells significantly suppresses Aβ production. NgR may limit the access of processing secretases to their APP substrate by either direct steric hindrance or an indirect mechanism.

Increased Aβ accumulation in mice lacking NgR

To examine the significance of the NgR/APP interaction on APP processing *in vivo*, the APPswe/PSEN-1(ΔE9) double-transgenic mouse (Borchelt et al., 1997; Jankowsky et al., 2003) was bred onto a NgR null background (Fig. 6). Brain sections and tissue extracts were examined for Aβ at 6 months of age. Compared with littermate-matched control mice, the absence of NgR significantly increases the accumulation of both Aβ plaque and immunoreactive Aβ (Fig. 6*a–c*). The similar elevations in $A\beta_{1-40}$ and $A\beta_{1-42}$ suggest that γ-secretase site preference is not altered by the absence of NgR.

One consequence of Aβ deposition is the formation of dystrophic neurites in and around plaques. To determine whether NgR absence might alter dystrophic neurite formation separately from Aβ accumulation, serial sections of the hippocampal dentate gyrus were examined for anti-synaptophysin immunoreactivity (Fig. 6*d*,*e*). The twofold increased Aβ plaque density of the $NgR^{-/-}$ animals is mirrored by a comparable increase in neuritic dystrophy. Thus, NgR modulates neuritic dystrophy in parallel with Aβ levels. We conclude that endogenous NgR has a role in restricting brain Aβ accumulation.

NgR(310)ecto-Fc treatment reduces Aβ plaque deposition

To increase NgR/APP interactions *in vivo*, soluble NgR(310)ecto-Fc protein was infused into APPswe/PSEN-1(ΔE9) double-transgenic mice (Borchelt et al., 1997; Jankowsky et al., 2003). The NgR(310)ecto-Fc protein contains the entire leucine-rich repeat ligand-binding domain of the NgR fused to the Fc portion of IgG (Lee et al., 2004; Li et al., 2004). To assess the specificity of action of this protein in brain, we examined the distribution of binding sites for NgR(310)ecto-Fc in wild-type and APPswe/PSEN-1(ΔE9) double-transgenic mouse brain by virtue of the rat Fc moiety (Fig. 7). The NgR(310)ecto-Fc protein, but not control IgG, associates with myelinated fiber tracts and prominently labels the corpus callosum and intraparenchymal fiber tracts in wild-type brain (Fig. 7*a*). This is consistent with binding to the previously described myelin ligands of NgR. In transgenic brain, the protein also labels Aβ-positive plaques (Fig. 7*b*). Whereas control IgG itself exhibits some detectable nonspecific binding for dense Aβ plaques, NgR(310)ecto-Fc binding is also detectable at the border of plaques in which lower levels of Aβ are found (Fig. 7*c*).

NgR(310)ecto-Fc was administered intracerebroventricularly by continuous infusion from 6- to 8-month-old mice with an Alzet minipump (Alza). Control APPswe/PSEN-1(ΔE9) mice received rat IgG because both the NgR and the Fc moiety were of rat origin. The total dose of protein infused was 0.4 mg/mouse over 56 d, corresponding to 0.29 mg/kg body weight per day. After

56 d, mice were killed, and the distribution of NgR(310)ecto-Fc and rat IgG was assessed (Fig. 8). The pattern of *in vivo* binding after intracerebroventricular infusion matches that for NgR(310)ecto-Fc binding to tissue sections. Myelinated tracts are labeled in white matter and in gray matter throughout the forebrain, demonstrating that the infused protein has access to the parenchyma of the brain. Some infused IgG is trapped in dense Aβ plaques, but specific NgR(310)ecto-Fc binding to the plaque periphery exceeds the nonspecific trapping of control protein.

For these treated mice, brain Aβ levels were measured by ELISA, and Aβ deposition into amyloid plaques was assessed by anti-Aβ immunohistochemistry. In the NgR(310)ecto-Fc mice, the deposition of immunoreactive Aβ into plaque is significantly reduced (Fig. 9*a*,*b*). The total level of both $A\beta_{1-40}$ and $A\beta_{1-42}$ decreases by 50% in the brain (Fig. 9*c*). The ratio of $A\beta_{40}$ to $A\beta_{42}$ is not altered in the treated group, arguing that γ-secretase function was not significantly modulated by soluble NgR. There is a tight correlation between Aβ levels and amyloid plaque deposition, suggesting that NgR(310)ecto-Fc alters APP/Aβ metabolism to a greater extent than Aβ aggregation itself (Fig. 9*d*).

Because dystrophic neurite formation occurs in and around amyloid plaques and NgR can regulate neurite growth, we considered whether NgR(310)ecto-Fc also modulates the formation of dystrophic neurites in APPswe/PSEN-1(ΔE9) mice. In the same group of mice, clusters of dystrophic neurites were identified by anti-synaptophysin immunohistology (Fig. 9*e*). Neuritic plaques are most prominent in the hippocampus, and the area occupied by synaptophysin-positive dystrophic neurite clusters is reduced in the NgR(310)ecto-Fc-treated transgenic mice (Fig. 9*f*). Microscopically, the morphology of dystrophic neurites formed in NgR(310)ecto-Fc-treated mice is similar to that in control familial AD (FAD) transgenic mice. Thus, excess NgR protein reduces both neuritic dystrophy and Aβ plaque deposition in FAD transgenic mice.

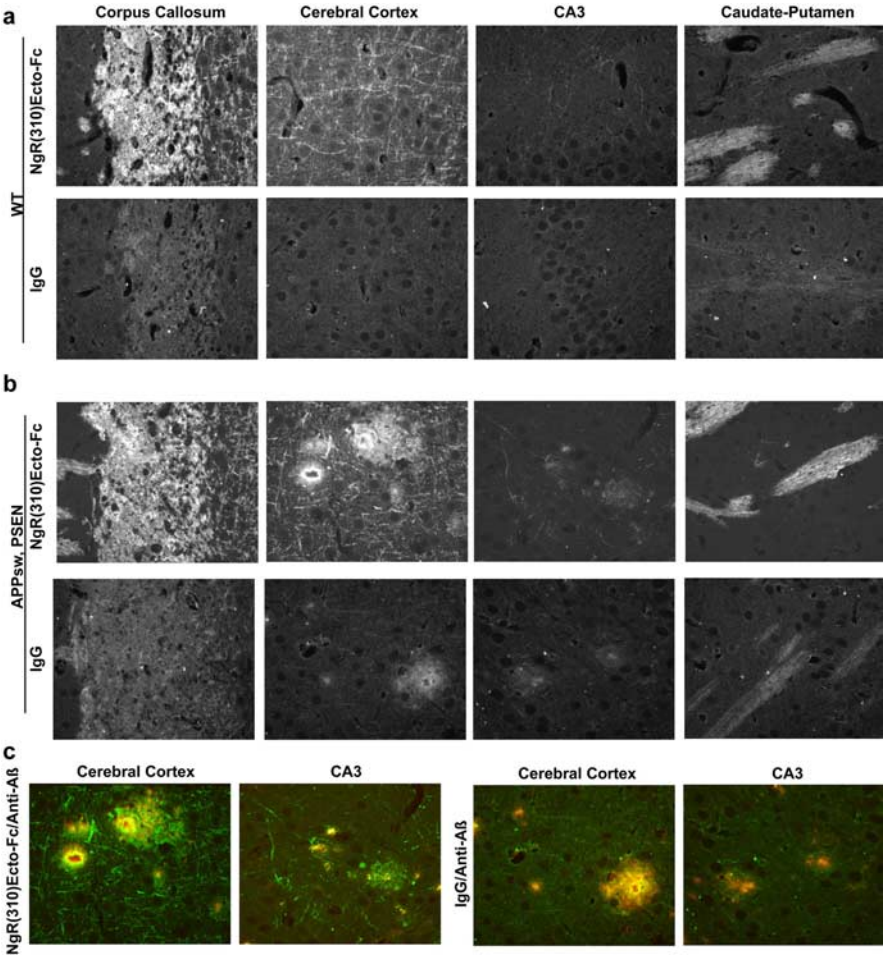

Figure 7. NgR(310)ecto-Fc binding sites in myelinated tracts and Aβ plaque in brain sections. ***a***, The binding of 0.1 μg/μl NgR(310)ecto-Fc or rat IgG to adult mouse brain sections was detected by fluorescent anti-rat IgG. Note the specific staining of myelinated tracts, prominent in corpus callosum and caudate–putamen. Intracortical and hippocampal fibers are labeled to a lesser degree. Brains were fixed in paraformaldehyde, sectioned in paraffin, and treated with formic acid before staining. ***b***, In brain sections from APPsw/PSEN-1 double-transgenic mice of 9 months of age, the pattern of myelinated tract staining by NgR(310)ecto-Fc but not IgG is similar to that in the wild-type (WT) animals of ***a***. At this concentration, NgR(310)ecto-Fc, and to a lesser extent IgG, also binds to plaque-like structures. ***c***, Double immunohistochemistry of rat NgR(310)ecto-Fc or rat IgG (green) with anti-Aβ (6E10, red). Both rat NgR(310)ecto-Fc and rat IgG bind to dense Aβ-positive plaque (yellow), but NgR(310)ecto-Fc is also enriched at zones of lower Aβ at the edges of plaques (adjacent green).

NgR causes parallel changes in sAPPα, sAPPβ, and Aβ

One mechanism by which excess NgR could reduce Aβ levels is by preventing α- and β-secretase access to APP substrate. A prediction from this model is that NgR-induced alterations in Aβ will be matched by changes in sAPP levels. In contrast, if NgR were to selectively alter Aβ clearance, then sAPP levels would not change when NgR levels are manipulated. If increased NgR were to favor α- over β-secretase cleavage of APP, then sAPPα versus sAPPβ/Aβ levels would exhibit opposite modulation by NgR.

To test these alternatives, we measured the α-secretase product sAPPα by immunoprecipitation and immunoblot after cell culture and *in vivo* manipulations of NgR (Fig. 10). The addition of NgR(310)ecto-Fc to APPswe-N2A cultures results in a reduction in sAPPα levels in the conditioned medium (Fig. 10*a*,*b*). *In vivo*, brains of NgR(310)ecto-Fc treated APPswe/PSEN-1(ΔE9) transgenic mice also exhibit decreased sAPPα levels (Fig. 10*c*,*d*). These reductions in sAPPα parallel the decreased Aβ level associated with elevated NgR (Figs. 5, 9). Levels of sAPPα are increased in the brain of APPswe/PSEN-1(ΔE9) transgenic mice lacking NgR (Fig. 9*e*,*f*), matching the increase of Aβ levels in these mice (Fig. 6). Levels of sAPPβ were also assessed in the transgenic mouse brain samples from knock-out mice with an antibody that selectively detects the C terminus of the transgenic sAPPβ after cleavage by β-secretase. Genetic ablation of NgR expression increases sAPPβ, although the magnitude of the increase is not as great as for sAPPα (Fig. 10*g*,*h*). Immunoblot detection of the C-terminal APP fragments (CTF) resulting from α- or β-secretases revels an increase in β-CTF levels; α-CTF levels could not be quantitated reliably (Fig. 9*i*,*j*). Because NgR regulates various APP fragments and Aβ levels in parallel, we conclude that NgR/APP association reduces cleavage of APP by both α- and β-secretases.

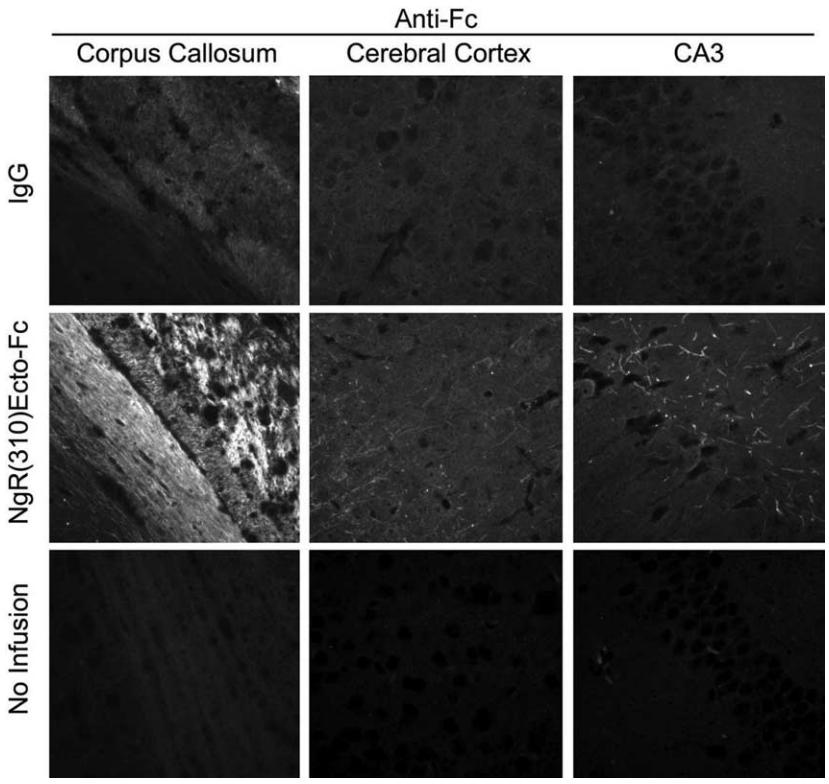

Figure 8. Localization of NgR(310)ecto-Fc after intracerebroventricular infusion. Brain sections were prepared from APPsw/PSEN-1 double-transgenic mice of 7 months of age after infusion with rat IgG, rat NgR(310)ecto-Fc, or no protein. The distribution of the infused protein was detected with fluorescently tagged anti-rat Fc and is similar to that of *in vitro* protein binding in Figure 6.

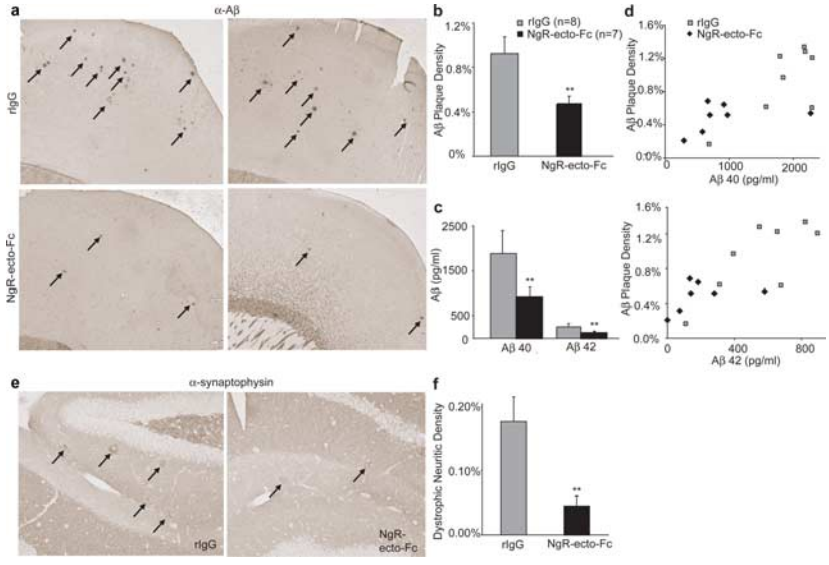

Figure 9. NgR-ecto-Fc decreases Aβ plaque formation and dystrophic neurites *in vivo*. Transgenic mice expressing both the human APPswe and PSEN-1(ΔE9) protein were treated from 6–8 months of age with either rat IgG or rat NgR(310)ecto-Fc from a minipump connected to an intracerebroventricular catheter. Each mouse received 0.4 mg of protein over 2 months (0.29 $mg \cdot kg^{-1} \cdot d^{-1}$). ***a***, ***b***, Transgenic mice were processed for Aβ immunohistology to reveal amyloid plaque deposition at 8 months of age (***a***). The arrows indicate examples of plaque deposits in sections of the cerebral cortex from three representative mice of eight or seven mice in each group. The area of cerebral cortex occupied by plaque was quantitated (***b***). Data are means ± SEM from $n = 8$ or 7 mice. The decrease in Aβ-positive plaques in the NgR(310)ecto-Fc group compared with control is significant (**$p \leq 0.02$, Student's *t* test). ***c***, Aβ was measured in formic acid extracts of brain. Data are means ± SEM from nine animals in each group. The decrease in Aβ in the NgR(310)ecto-Fc group compared with IgG is significant (**$p \leq 0.02$, Student's *t* test). The ratio of $A\beta_{40}$ to $A\beta_{42}$ is not altered in the NgR(310)ecto-Fc-treated group. ***d***, Correlation of plaque density and $A\beta_{40}$ or $A\beta_{42}$ levels in mice treated as in ***a–c***. ***e***, ***f***, Anti-synaptophysin-immunoreactive dystrophic neurites in control and NgR(310)ecto-Fc-treated transgenic hippocampus are illustrated (arrows, ***e***). The percentage of area occupied by synaptophysin-positive dystrophic neurites is reported in ***f***. Data are means ± SEM from eight or seven mice per group. **$p < 0.02$, Student's *t* test; the decrease in dystrophic neurites with NgR(310)ecto-Fc treatment is significant.

Discussion

Several lines of evidence presented here indicate that the NgR protein plays a significant role in APP/Aβ pathophysiology. Neuronal NgR localization is altered in AD brain. NgR protein physically associates with APP. Endogenous NgR/APP/Aβ interaction serves to suppress the production of Aβ and the Aβ plaque deposition that is characteristic of AD. Alterations of NgR function by gene targeting or by infusion of soluble NgR(310)ecto-Fc regulate Aβ levels in transgenic mouse brain. There is an inverse relationship between the level of NgR and the level of Aβ, plaque deposits, and neuritic dystrophy. Elimination of NgR expression increases these measures of AD activity, whereas treatment with excess soluble NgR protein reduces Aβ, plaques, and neuritic dystrophy.

The metabolism of APP and Aβ consists of numerous steps that must be considered in explaining the effects of NgR on Aβ. Certain steps are unlikely to play a significant role based on the current studies. Because Aβ levels, amyloid plaque deposition, and neuritic dystrophy change in parallel, it is unlikely that Aβ aggregation or the induction of dystrophy is strongly altered by NgR. The extracellular location of NgR and the absence of any alteration in $A\beta_{40}/A\beta_{42}$ ratio with NgR manipulations argue that γ-secretase is not a major site of NgR modulation of APP/Aβ pathology. Exogenous soluble NgR(310)ecto-Fc appears to enhance endogenous NgR action, acting in opposition to NgR gene deletion. Therefore, NgR-mediated signaling for axon outgrowth inhibition is unlikely to play a central role for the ability of NgR to reduce Aβ pathology. If, instead, NgR signaling within neurons were crucial, then the soluble decoy receptor would have identical (not opposite) effects to those of NgR gene disruption. It is also unlikely that the effects of extracellular NgR(310)ecto-Fc on Aβ pathology can be explained primarily by altered intracellular APP trafficking. Could the formation of an Aβ complex with cell surface endogenous NgR or extracellular NgR(310)ecto-Fc alter the degradation or clearance of the peptide? Specifically, might exogenous NgR(310)ecto-Fc lower Aβ by acting as a "sink" to remove Aβ in the same manner as does anti-Aβ antibody? The limiting effect of endogenous NgR on Aβ pathology and the ability of NgR overexpression in neuroblastoma cells to reduce Aβ suggest that a sink hypothesis does not explain the principal action of NgR on Aβ pathology.

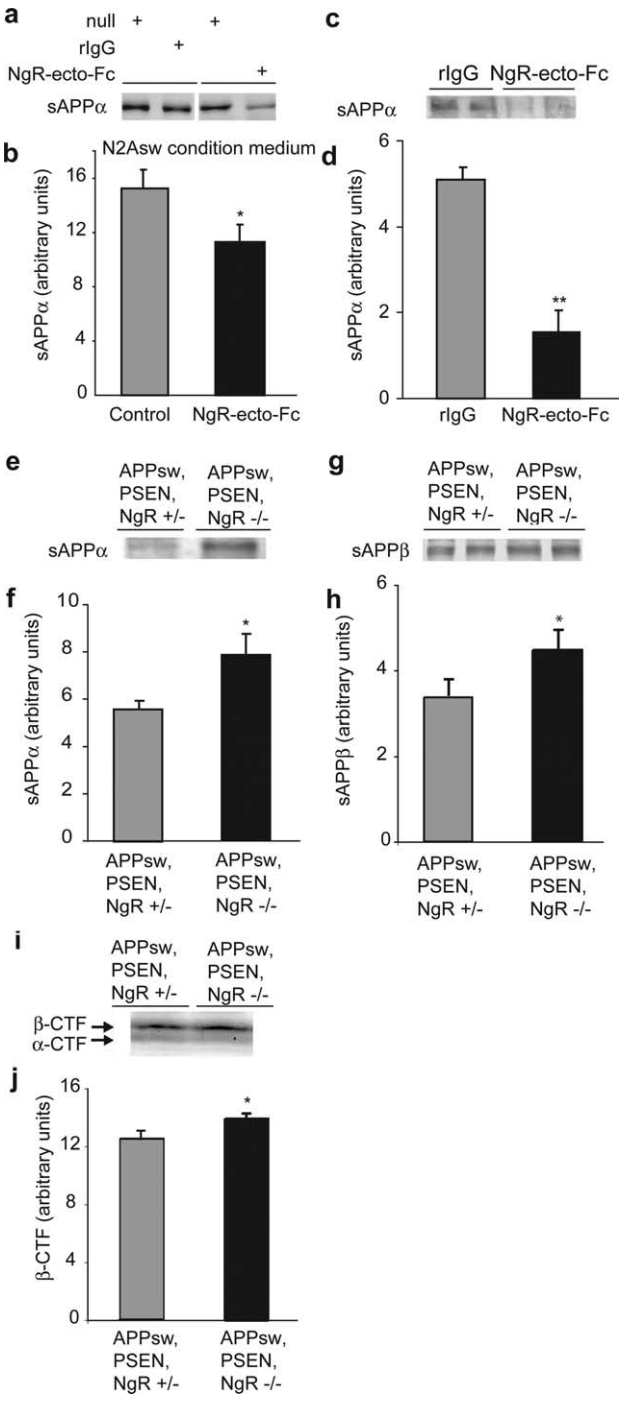

Figure 10. NgR regulates sAPPα in parallel with Aβ levels. The level of sAPPα in brain extracts or N2A culture medium was analyzed by immunoprecipitation with anti-N-terminal-APP 22C11 antibody and immunoblot with anti-$A\beta_{1-17}$ 6E10 antibody. The sAPPβ fragment was analyzed in brain tissue by direct immunoblots with anti-sAPPβswe C-terminal antibody, and the CTFs were detected by the anti-APP-C-terminal 369 antibody. Immunoreactivity per milligram of protein was assessed in independent samples for $n = 6–9$ mice or cell cultures in each experimental group (mean ± SEM; $*p < 0.05$ level, $**p < 0.01$, Student's *t* test). ***a***, ***b***, sAPPα levels in conditioned medium from APPswe-expressing N2A cells was quantitated after 48 h culture in the presence of 1 mg/ml control rat IgG or rat NgR(310)ecto-Fc. ***c***, ***d***, sAPPα in brain extracts from APPswe/PSEN-1(ΔE9) transgenic mice treated from 6–8 months of age with either rat IgG or rat NgR(310)ecto-Fc intracerebroventricularly, as in Figure 9. ***e***, ***f***, sAPPα in brain extracts from 6-month-old littermate-matched mice transgenic for APPswe/PSEN-1(ΔE9) and either $NgR^{+/-}$ or $NgR^{-/-}$, as in Figure 6. ***g***, ***h***, sAPPβ in brain extracts from 13-month-old littermate-matched mice transgenic for APPswe/PSEN-1(ΔE9) and either $NgR^{+/-}$ or $NgR^{-/-}$. ***i***, ***j***, α-CTF and β-CTF in brain extracts from 13-month-old littermate-matched mice transgenic for APPswe/PSEN-1(ΔE9) and either $NgR^{+/-}$ or $NgR^{-/-}$. The β-CTF level is quantitated in ***j***.

These considerations imply that NgR blockade of initial α-secretase and β-secretase cleavage of APP is likely to be a principal site of NgR action *in vivo*. Because NgR binds to the extracellular region of APP that serves as a secretase substrate, NgR might serve as a competitive inhibitor of processing. Alternatively, the APP/NgR interaction may indirectly reduce APP access to secretase compartments in the cell. Consistent with NgR-mediated blockade of both α- and β-secretase access to APP, we found that the levels of the sAPPα, sAPPβ, and β-CTF fragment are altered in parallel with Aβ levels in $NgR^{-/-}$ mice and NgR(310)ecto-Fc-treated mice. Together, the data are consistent with the hypothesis that NgR reduces Aβ pathology primarily by reducing α- and β-secretase cleavage.

Apart from Aβ pathophysiology and AD, NgR interactions with APP and Aβ may play roles in normal neuronal physiology. Because APP has been identified as an adaptor for kinesin-dependent transport of certain cargos in the axon (Kamal et al., 2001), NgR localization to the distal axon may depend on binding to APP. Studies of NgR distribution and function in APP null mice should resolve this issue. Although we did not observe direct competition between Aβ and Nogo-66, myelin-associated glycoprotein (MAG), or oligodendrocyte myelin glycoprotein for NgR binding (data not shown), there may be modulatory effects of APP or Aβ on axon growth inhibition by CNS myelin under certain conditions distinct from those examined in preliminary experiments. Because Aβ has been implicated as a regulator of synaptic function recently (Walsh et al., 2002b; Kamenetz et al., 2003), NgR may participate in this modulation directly or indirectly. Overall, the association of APP and NgR tightens the links between axonal function and the neurodegenerative process in AD.

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